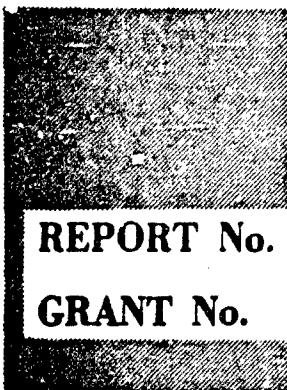


AD710330



AD

REPORT No. FE-428-2 (Final Report)

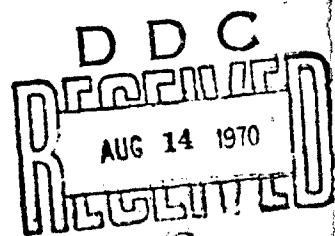
GRANT No. DA-CRD-AFE-S92-544-69-G153

STRUCTURE-ACTIVITY RELATIONSHIPS AND IMMUNOCHEMICAL STUDIES  
ON COBROTOXIN

by

Chen-chung Yang, D. Med. Sc.

Professor of Biochemistry  
Kaohsiung Medical College  
Kaohsiung, Taiwan  
Republic of China



July 1970

Reproduced by the  
CLEARINGHOUSE  
for Federal Scientific & Technical  
Information Springfield Va. 22151

U. S. ARMY RESEARCH AND DEVELOPMENT GROUP  
FAR EAST  
APO San Francisco 96343



ACCESSION BY	
CGSY	WHITE SECTION <input checked="" type="checkbox"/>
DDG	BUFF SECTION <input type="checkbox"/>
UNARMEDGED	
JUSTIFICATION	
BY	
DISTRIBUTION/AVAILABILITY CODES	
DIST.	AVAIL. DDG. OR SPECIAL
/	

#### **DDC DISTRIBUTION AND AVAILABILITY NOTICE**

This document has been approved for public release and sale; its distribution is unlimited.

#### **DISPOSITION INSTRUCTIONS**

Destroy this report when it is no longer needed. Do not return it to the originator.

The findings in this report are not to be construed as an official Department of the Army position unless so designated by other authorized documents.

REPORT No. FE-428-2 (Final Report)

GRANT No. DA-CRD-AFE-592-544-69-G153

DA Project/Task/Work Unit No. 2N061102B71D CO 128FE

STRUCTURE-ACTIVITY RELATIONSHIPS AND IMMUNOCHEMICAL STUDIES  
ON CORROTOXIN

by

Chen-chung Yang, D. Med. Sc.

Professor of Biochemistry  
Kaohsiung Medical College  
Kaohsiung, Taiwan  
Republic of China

July 1970

U. S. ARMY RESEARCH AND DEVELOPMENT GROUP  
FAR EAST  
APO San Francisco 96343

---

This document has been approved for public  
release and sale; its distribution is unlimited.

STRUCTURE-ACTIVITY RELATIONSHIPS AND IMMUNOCHEMICAL STUDIES ON COBROTOXIN

The Position of Disulfide Bonds in Cobrotoxin

The amino acid sequence of cobrotoxin has recently been established in our laboratory. However, in consideration that the disulfide bonds are the major bonds which maintain the protein in its native configuration and that the integrity of the disulfide bonds is important for the venom toxicity, it is therefore essential that each pair of the half-cystinyl residues is determined, to assist in the elucidation of the two-dimensional structure of cobrotoxin.

Cobrotoxin was digested with acid protease A and the resulting five cystine peptides were separated by high-voltage paper electrophoresis. The identification of the disulfide bridges was made by determining the amino acid composition of the corresponding cysteic acid peptides obtained after the oxidation of the single cystine peptides. The double cystine peptide which contains the -Cys-Cys- linkage in sequence from the acid protease A digest was further partially hydrolyzed with acid under conditions in which the disulfide bonds were stable. Five cystine peptides were obtained from which the two remaining disulfide bridges were established. The specificity of the acid protease A, the selectivity of acid hydrolysis, and the structure of cobrotoxin were discussed. (1)

5407

ABSTRACT

The Position of Disulfide Bonds in Cobrotoxin

The amino acid sequence of cobrotoxin has recently been established in our laboratory. However, in consideration that the disulfide bonds are the major bonds which maintain the protein in its native configuration and that the integrity of the disulfide bonds is important for the venom toxicity, it is therefore essential that each pair of the half-cystinyl residues is determined, to assist in the elucidation of the two-dimensional structure of cobrotoxin.

The fragmentation of protein in the study of disulfide bridges is usually carried out with pepsin under acidic pH. However, cobrotoxin is not attacked by pepsin and is also not cleaved to single cystine peptides by either trypsin or chymotrypsin or the combination of both. Consequently, the fragmentation of the toxin was carried out with acid protease A.

Cobrotoxin was digested with acid protease A in 5 % acetic acid and the resulting S-containing peptides were separated by high-voltage paper electrophoresis. The identification of the disulfide bridges was made by determining the amino acid composition of the corresponding cysteic acid peptides obtained after the oxidation of the single cystine peptides. However, peptide AII gave the result indicating that it represents the sequence from positions (42)-43 to 61-(62) of cobrotoxin. Hence it is a double cystine peptide which contains the -CyS-CyS- linkage in sequence was not cleaved by the acid protease A. Therefore, the peptide was further subjected to partial acid hydrolysis at 37° for 10 days with 10 N H<sub>2</sub>SO<sub>4</sub> in 50 % acetic acid containing thioglycollic acid. Five cystine peptides were obtained from which the two remaining disulfide bridges were established.

The result of this study was summarized in Fig. 4, which gave the complete structure of cobrotoxin showing the position of the disulfide bonds and the sequence of the amino acid residues. The region of the molecule from residues 25 to 40 contains most of the basic and the functional residues in close order and is completely free of prolyl and cystinyl residues. This uncross-linked loop, projecting outward from the molecule because of its hydrophilic character. It is the only region in the molecule where potentially, a considerable degree of  $\alpha$ -helical structure could be present and may play an important role in toxicity.

Table of Contents

<b>The Position of Disulfide Bonds in Cobrotoxin</b>	<b>Page 1</b>
<b>I. Introduction</b>	<b>1</b>
<b>II. Materials and Methods</b>	<b>1</b>
1. Digestion of cobrotoxin	1
2. Isolation of cystine peptides	1
3. Oxidation of cystine peptides	2
4. Amino acid analysis	2
5. Partial acid hydrolysis of the double cystine peptide which contains the -CyS-CyS- linkage	2
<b>III. Results</b>	<b>2</b>
1. Digestion of cobrotoxin by acid protease A	2
2. Partial acid hydrolysis of the double cystine peptide IIIa	5
<b>IV. Discussion</b>	<b>6</b>
<b>V. Conclusion</b>	<b>8</b>
<b>Literature Cited</b>	<b>9</b>
<b>Appendices</b>	<b>10</b>
<b>List of Publications</b>	<b>16</b>
<b>List of Graduate Students</b>	<b>17</b>
<b>Distribution List</b>	
<b>DD Form 1473</b>	

List of Appendixes

A-1 Table I. Amino acid composition and the most possible sequence of cysteic acid peptides from acid protease A digest of cobrotoxin. Page 10

A-2 Table II. Amino acid composition and probable sequence of cysteic acid peptides obtained from acid hydrolysates of the double cystine peptide AIIa. 11

B-1 Fig. 1. Electrophoretic separation of cystine peptides from acid protease A digest of cobrotoxin (pH 5.4, 48 V/cm, 90 min) and of cysteic acid peptides from the cystine peptides (pH 5.4, 48 V/cm, 60 min). 12

B-2 Fig. 2. Electrophoresis of the neutral band AII at pH 1.9 and the separation of cysteic acid peptides from AIIa (pH 1.9, 56 V/cm, 90 min). 13

B-3 Fig. 3. Electrophoretic separation of (A) cystine peptides from acid hydrolysates of AIIa (pH 5.4, 46 V/cm, 90 min) and of (B) the oxidation products of the cystine peptides (pH 5.4, 46 V/cm, 60 min). 14

B-4 Fig. 4. Structure of cobrotoxin. Two-dimensional schematic diagram showing the arrangement of the disulfide bonds and the sequence of the amino acid residues. 15

## The Position of Disulfide Bonds in Cobrotoxin

### I. Introduction

The amino acid sequence of cobrotoxin has recently been established in our laboratory (1). However, in consideration that the disulfide bonds are the major bonds which maintain the protein in its native configuration (2, 3), and that the integrity of the disulfide bonds is important for the venom toxicity (4), it is therefore essential that each pair of the half-cystinyl residues is determined, to assist in the elucidation of the two-dimensional structure of cobrotoxin.

Considerable work has been done successfully with this problem (5-8) and the diagonal electrophoretic technique of Brown and Martley (7, 8) has been proved to be a very convenient method. The experiment to be described indicates that the separation of cystine and cysteic acid peptides from the digest of cobrotoxin was achieved by this method and of Ryle *et al.* (5), and that the partial fragmentation of cobrotoxin was carried out successfully by using the combination of acid protease A and acid hydrolysis under the condition used by Ryle *et al.* (5).

The identification of disulfide bonds was made by determining the amino acid composition of the corresponding cysteic acid peptides. From the results of this investigation, the four disulfide bonds in cobrotoxin have been established.

### II. Materials and Methods

Cobrotoxin used in this study was prepared from Formesan cobra (*Naja naja atra*) venom as previously described (9). Acid protease A, PAP-2, was the product of Seikagaku Kogyo Company, Japan. Dowex 2-X 8 (acetate form, 200-400 mesh) was obtained from Bio-Rad Company and thioglycollic acid was purchased from Sigma Chemical Company. All other reagents were of analytical reagent grade.

#### 1. Digestion of cobrotoxin

Cobrotoxin was dissolved in 5 % acetic acid (pH 2.3) to make a 1 % solution and acid protease A (10 : 1) was added. Digestion was performed at 37° for 24 h and the digest was taken to dryness under vacuum in a desiccator.

#### 2. Isolation of cystine peptides

Cystine peptides from acid protease A digest were separated

by high-voltage paper electrophoresis at pH 5.4 with pyridine-acetic acid-water (20 : 7 : 973, by vol.) (1) on a 30 x 74 cm sheet of Whatman No. 3 MM paper. After drying, 1 cm strips were cut out from both sides of the electrophoretogram and developed with CN-nitroprusside reagent (10). The main neutral band was cut out and stitched to a fresh sheet for the second electrophoretic purification at pH 1.9. All cystine peptide bands were cut out, eluted with 1 M acetic acid and the eluates were subjected to oxidation with performic acid.

### 3. Oxidation of cystine peptides

The oxidation was carried out by adding a few drops of a solution of performic acid freshly prepared by mixing 1 vol. of 33 %  $H_2O_2$  with 9 vol. of formic acid. Oxidation was allowed to proceed for 30 min, a few drops of water were then added and the solutions were taken to dryness. The residues were subjected to paper electrophoresis at pH 5.4 and the cysteic acid peptides were developed with 0.2 % ninhydrin in acetone.

### 4. Amino acid analysis

The cysteic acid peptide bands cut out from the electrophoretogram were eluted with constant boiling HCl (5.7 N) and hydrolyzed at 110° for 24 h in evacuated sealed tubes. The amino acids were determined on a Technicon amino acid autoanalyzer using a 0.3 x 75 cm column and 50 mm flow cell to increase the sensitivity to detect amino acids in concentrations as low as 5 nanomoles.

### 5. Partial acid hydrolysis of the double cystine peptide which contains the -Cys-Cys- linkage

The peptide was partially hydrolyzed at 37° for 10 days with 10 N  $H_2SO_4$  in 50 % acetic acid (2 ml) containing thioglycollic acid (1.32 mg). The hydrolysate was freed from  $H_2SO_4$  by passage through a column of Dowex 2-X 8 using 20 % acetic acid as an eluent. The eluate was evaporated to dryness and the residue was subjected to electrophoresis for the identification of cystine and cysteic acid peptides as described above.

## III. Results

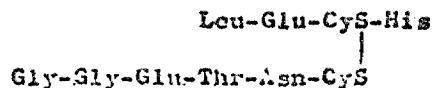
### 1. Digestion of cobrotoxin by acid protease A

The fragmentation of protein in the study of disulfide bridges is usually carried out with pepsin under acidic pH. However, cobrotoxin is not attacked by pepsin (11) and is also not cleaved to single cystine peptides by either trypsin or chymotrypsin or the

combination of both (1). Consequently, the fragmentation of the toxin was carried out with acid protease A.

Fig. 1 shows the distribution of cystine peptides after electrophoresis of the acid protease A digest of cobrotoxin at pH 5.4. Five peptide bands were obtained, of which the neutral band AII was refractionated by electrophoresis at pH 1.9. As shown in Fig. 2, the band was resolved into one major band AIIa and two minor bands AIIb and AIIc. All cystine peptides were oxidized by performic acid and the resulting cysteic acid peptides were separated by electrophoresis at the same pH (Figs. 1 and 2). The cysteic acid peptides sufficient enough to give results were eluted for amino acid analysis. The amino acid composition and the most possible sequence of the peptides are presented in Table I.

Band AI: Five cysteic acid peptides were resulted from this fraction as shown in Fig. 1. AIIa gave an initial yellow color in ninhydrin reaction and had an amino acid composition of  $\text{CySO}_3\text{H}_1$ ,  $\text{Asp}_1$ ,  $\text{Thr}_1$ ,  $\text{Glu}_1$ ,  $\text{Gly}_2$  which could only represents the sequence of Gly-Gly-Glu-Thr-Asp-CySO<sub>3</sub>H in cobrotoxin. Aspartic acid, not asparagine, was present in this peptide as comparing the mobility with AIIb. The occurrence of aspartic acid in this peptide indicates that asparagine originally present at the position had partially converted to aspartic acid during 24 h incubation at 37° in 5% acetic acid. AIIb gave the composition as indicated in Table I, suggesting that it is a mixture of two peptides, one of which is AIIc since it ran partly overlapped with this peptide. Upon deduction, the other peptide must be the sequence of Gly-Gly-Glu-Thr-Asn-CySO<sub>3</sub>H, which differs from AIIa only in having one amide group and therefore being less acidic. AIIc was determined to be the sequence of Leu-Glu-CySO<sub>3</sub>H-His, since these four amino acids do not occur together elsewhere in the molecule. AIId and AIIe were still impure at this stage, and since these peptides had no cysteic acid, no attempt was made to purify them. From the composition of peptides AIIa and AIIc, it clearly indicates that the two cysteic acid peptides arose from a single cystine peptide of

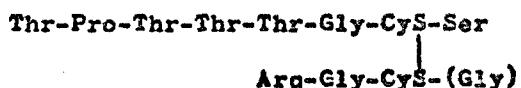


Band AIII: It is a neutral peptide at pH 5.4 and can be separated into one major band and two minor bands at pH 1.9 (Fig. 2). On oxidation, the major band AIIIa resolved into at least eight cysteic acid peptides. The major cysteic acid peptides, AIIIa<sub>1</sub> and AIIIa<sub>2</sub>, were eluted for amino acid analyses without further purification. AIIIa<sub>1</sub> gave the result (Table I) indicating that it represents the sequence from the position (42-43) to 61(-62) of cobrotoxin. Hence it is a double cystine peptide, of which the

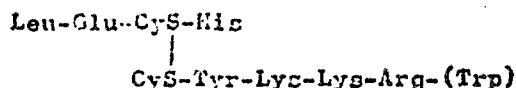
-CyS-CyS- linkage in sequence was not cleaved by acid protease A. AIIa<sub>2</sub> was still a complex mixture as indicated from its composition and may probably represent the same sequence with different enzymic cleavages. In order to establish the containing two disulfide bridges, AIIa was further subjected to partial acid hydrolysis as described later.

Band AIII: It was stained only weakly with CN-nitroprusside. AIIa gave the composition of CySO<sub>3</sub>H<sub>1</sub>, Asp<sub>2</sub> and Arg<sub>1</sub> on amino acid analysis and probably contained two aspartyl residues as judging from its mobility at pH 5.4. Therefore, the sequence of the peptide might be Arg-CySO<sub>3</sub>H-Asp-Asp. AIIIB and AIIIC were still impure at this stage. They may also represent the sequence from position 42 to 62 with a contamination of other cysteic acid peptides from different cystine peptides. Since these bands only represented a mixture of minor varieties of the sequence which contained at least three disulfide bonds, it was not further dealt with.

Band AIV: Only two cysteic acid peptides appeared on performic acid oxidation (Fig. 1). AIVa was stained initially yellow with ninhydrin and had one net negative charge at pH 5.4. The composition was determined as given in Table I which represents the sequence of Thr-Pro-Thr-Thr-Gly-CySO<sub>3</sub>H-Ser, since these residues only occur together in the sequence in cobrotoxin. AIVb was neutral and had the composition to be the sequence of Arg-Gly-CySO<sub>3</sub>H-(Gly). So it is clear that these two cysteic acid peptides arose from a single cystine peptide of



Band AV: Four cysteic acid peptides arose from this band on oxidation. AVa was identical with AIC. AVb and AVc had the same composition and represent the same sequence. However, AVc had one more lysyl residue than AVb, this may account for AVc which migrated more toward the cathode than AVb. AVd had an unknown spot (behind aspartic acid) on electrophoretogram at pH 1.9, it is probably suggestive of oxidized tryptophan. Thus, these cysteic acid peptides must be arose from a single cystine sequence by different enzymic cleavage and the cystine peptides would be

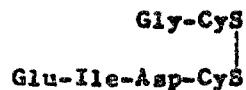


The presence of three basic residues may account for this peptide moving furthest toward cathode.

2. Partial acid hydrolysis of the double cystine peptide AIIa

Partial acid hydrolysis was performed with AIIa isolated from 400 mg of cobra toxin under the conditions described in Methods. Fig. 3-A shows the electrophoretic separation of cystine peptides from acid hydrolysates of AIIa. Band PI was considered too weak to give any results after oxidation, so it was not further dealt with. The remainder of the bands were oxidized with performic acid and separated by electrophoresis at pH 5.4 (Fig. 3-B). From their electrophoretic mobilities and composition, it is possible to deduce the structure of these cysteic acid peptides and the results are given in Table II.

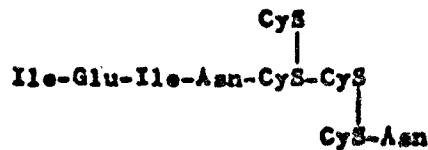
Band PII: Six cysteic acid peptides arose from this band. PII<sub>4</sub>, PII<sub>5</sub> and PII<sub>6</sub> represented the same sequence at different acid cleavage, in which aspartyl residue was present rather than asparaginyl as comparing the mobility with PII<sub>2</sub>. PII<sub>3</sub> which gave an initial yellow ninhydrin color had only CySC<sub>3</sub>H and Gly, indicating to be the sequence of Gly-CySO<sub>3</sub>H located at the positions 42 and 43. PII<sub>2</sub> and PII<sub>1</sub> were too weak to give results. Thus, all of these cysteic acid peptides must arose from the single cystine peptide of



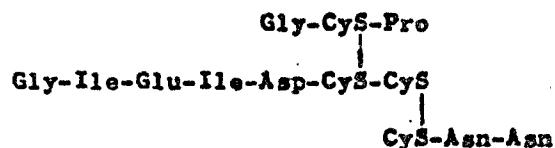
Band PIII: It gave five cysteic acid peptides. PIII<sub>5</sub> was probably a mixture of CySO<sub>3</sub>H-Asp and CySO<sub>3</sub>H, since both would expect to move at the same distance, whereas Asp-CySO<sub>3</sub>H-CySO<sub>3</sub>H moved faster as shown in PIV<sub>5</sub>. PIII<sub>4</sub> has a structure of Ile-Glu-Ile-Asn-CySO<sub>3</sub>H-CySO<sub>3</sub>H. As compared the mobility with PIV<sub>4</sub>, aspartyl residue would be present as an amide form. PIII<sub>3</sub> was a dipeptide of CySO<sub>3</sub>H-Asn, as judging from its mobility, and was probably not Asn-CySO<sub>3</sub>H from the positions 53 and 54, since the amide group would expect to go off before cleavage of the Ile-Asn linkage. PIII<sub>2</sub> and PIII<sub>1</sub> have the sequence of Glu-Ile-Asn-CySO<sub>3</sub>H and Gly-Ile-Glu-Ile-Asn-CySO<sub>3</sub>H, respectively. The former has the same composition as PII<sub>4</sub>, but with different mobility suggesting the presence of asparaginyl residue. Thus, these cysteic acid peptides probably arose from two cystine peptides. One of which is



and the ether is



Band PIV: It gave five cysteic acid peptides on performic acid oxidation.  $\text{PIV}_3$ ,  $\text{PIV}_4$  and  $\text{PIV}_5$  represented the same sequence at different acid cleavage and the -CyS-CyS- linkage still remained intact.  $\text{PIV}_2$  was identical with  $\text{PIII}_3$ .  $\text{PIV}_1$  was probably a mixture of two peptides  $\text{Gly-CySO}_3\text{H-Pro}$  and  $\text{CySO}_3\text{H-Asn-Asn}$ , since these residues should not occur together in the sequence. Therefore, the cystine peptide would be



Band PV: Three cysteic acid peptides arose from this band on oxidation.  $\text{PV}_1$  and  $\text{PV}_2$  were dipeptides of  $\text{CySO}_3\text{H-Pro}$  and  $\text{Gly-CySO}_3\text{H}$ , respectively.  $\text{PV}_3$  contained only  $\text{CySO}_3\text{H}$ . Therefore, the cystine peptides would probably be



Band PVI: This band yielded only cysteic acid after performic acid oxidation. So the band could be the free cystine which probably represents the residues at the positions 55 and 60, since the cleavage of the linkages CyS-Thr, CyS-Asn and Arg-CyS would be expected to be easier than those of -CyS-CyS- and Ile-Asn-CyS linkages.

#### IV. Discussion

The specificity of acid protease A for cobrotoxin was observed in this study. The enzyme was apparently to split the bonds that are amino terminal to arginine for peptides  $\text{AIIIA}$ ,  $\text{AIVb}$ ,  $\text{AVc}$ ,  $\text{AVd}$  and non-cysteic acid peptide  $\text{Arg-Gly-Tyr}$  (not recorded). With relatively high recoveries of peptides  $\text{AIc}$ ,  $\text{AIVa}$  and  $\text{AVa}$ , it also indicated to split the bonds that are carboxyl terminal to histidine or glutamine or serine. An interesting result was obtained with peptides  $\text{AIa-b}$  and  $\text{AVb-d}$ , in which the enzyme attacked either side of the cysteinyl residue. A similar result was also observed with peptide  $\text{AIVb}$ , in which the enzyme attacked one side of the

cystinyl residues at the positions 41 and 43.

The selectivity of partial acid hydrolysis under the condition used in this experiment was observed with the double cystine peptide AIIa which contains the -CyS-CyS- linkage in sequence. The largest peptide isolated from the hydrolysate was peptide PIV which strongly suggests that extensive cleavage at the linkages of Pro-Ser (44-45), Asn-Gly (48-49), CyS-Thr (55-56) and Arg-CyS (59-60) had occurred. The isolation of peptides PII and PIII in higher amount indicated that cleavage of the -CyS-CyS- linkage in sequence had occurred before the cleavage of linkages between 50 to 54 which is sterically hindered by two isoleucyl residues. The absence of cystinyl-aspartic acid also indicated that asparagine at the position 61 was released before the cleavage of -CyS-CyS- linkage. This would account for the fact that free cystine (PVI) was present in appreciable amount and that no cystine peptides were detected to confirm the position of the fourth disulfide bridge.

Of total cystine peptides isolated from the hydrolysate, about 60 % was obtained without the presence of -CyS-CyS- linkage in sequence, and most of the cysteinyl residue at the position 43 was still labeled with either glycyl or prolyl residue. This would enable us to establish the third disulfide bridge, and the fourth which must therefore be joined together.

The result of this study was summarized in Fig. 4, which gave the complete structure of cobrotoxin showing the position of the disulfide bonds and the sequence of the amino acid residues. It is clear that the sequence from the position 35 to 40 is exposed outwardly on the protein. This was anticipated as described in the preceding paper (1). An interesting feature of the structure is that it contains a ring, in which the disulfide bond in positions 55 to 60 is included. This ring is the same size as the similar disulfide ring found in oxytocin, vasopressin and insulin (5) which suggests that it may have a possible structural or biological significance.

It is also significant that cobrotoxin, having only 62 amino acid residues, contains four disulfide bonds and that its full activity depends the integrity of these four disulfide bonds (4). So that the conformation of cobrotoxin is very important to its biological functions. Since cobrotoxin is richly composed of the hydrophilic amino acids, it must be relied on the disulfide bonds to maintain its native conformation (2, 3). This may account for the fact that four disulfide bonds are present out of 62 residues, and the fact that, in the presence of 8 M urea, its activity is still fully retained (4).

#### V. Conclusion

The position of four disulfide bonds in cobretoxin was investigated. Cobretoxin was digested with acid protease A and the resulting five cystine peptides were separated by high-voltage paper electrophoresis. The identification of the disulfide bridges was made by determining the amino acid composition of the corresponding cysteic acid peptides obtained after the oxidation of the single cystine peptides. The double cystine peptide which contains the -CyS-CyS- linkage in sequence from the acid protease A digest was further partially hydrolyzed with acid under conditions in which the disulfide bonds were stable. Five cystine peptides were obtained from which the two remaining disulfide bridges were established. The specificity of the acid protease A, the selectivity of acid hydrolysis, and the structure of cobretoxin were discussed.

Literature Cited

1. C. C. Yang, H. J. Yang and J. S. Huang, Biochim. Biophys. Acta, 188 (1969) 65.
2. C. C. Yang, C. C. Chang, K. Hamaguchi, K. Ikeda, K. Hayashi and T. Suzuki, J. Biochem., 61 (1967) 272.
3. C. C. Yang, C. C. Chang, K. Hayashi, T. Suzuki, K. Ikeda and K. Hamaguchi, Biochim. Biophys. Acta, 168 (1968) 373.
4. C. C. Yang, Biochim. Biophys. Acta, 133 (1967) 346.
5. A. L. Ryle, F. Sanger, L. P. Smith and R. Kitai, Biochem. J., 60 (1955) 541.
6. A. P. Ryle and C. B. Anfinsen, Biochim. Biophys. Acta, 24 (1957) 633.
7. J. R. Brown and B. S. Hartley, Biochem. J., 101 (1966) 214.
8. J. R. Brown, D. L. Kauffman and B. S. Hartley, Biochem. J., 103 (1967) 497.
9. C. C. Yang, J. Sci. Chem., 240 (1965) 1616.
10. G. Teennies and J. J. Kolb, Anal. Chem., 23 (1951) 829.
11. C. C. Yang, Texicon, 3 (1965) 19.

## APPENDIX A-1

Table I

Amino acid composition and the most possible sequence of cysteic acid peptides from acid protease A digest of cobrotoxin

Peptides	Amino acid composition	Probable sequence of the peptides
AIa	CySO <sub>3</sub> H <sub>1.0</sub> , Asp <sub>1.0</sub> , Thr <sub>0.9</sub> , Glu <sub>0.6</sub> , Gly <sub>1.2</sub>	Gly-Gly-Glu-Thr-Asp-
AIb	CySO <sub>3</sub> H <sub>2.3</sub> , Asp <sub>0.9</sub> , Thr <sub>1.0</sub> , Glu <sub>1.7</sub> , Gly <sub>1.2</sub> Leu <sub>1.3</sub> , His <sub>1.3</sub>	Leu-Glu-CySO <sub>3</sub> H-His Gly-Gly-Glu-Thr-Asp-
AIc	CySO <sub>3</sub> H <sub>1.1</sub> , Glu <sub>1.0</sub> , Leu <sub>0.8</sub> , His <sub>1.0</sub>	Leu-Glu-CySO <sub>3</sub> H-His
-----	-----	-----
AIId <sub>1</sub>	CySO <sub>3</sub> H <sub>3.6</sub> , Asp <sub>3.9</sub> , Thr <sub>2.0</sub> , Ser <sub>1.2</sub> , Glu <sub>1.0</sub> , Gly <sub>1.5</sub> , Val <sub>0.7</sub> , Ile <sub>1.8</sub> , Lys <sub>1.0</sub> , Arg <sub>1.0</sub>	(42)-43 61-(6)
AIId <sub>2</sub>	CySO <sub>3</sub> H <sub>4.6</sub> , Asp <sub>6.1</sub> , Thr <sub>2.1</sub> , Ser <sub>1.0</sub> , Glu <sub>1.0</sub> , Gly <sub>1.0</sub> , Val <sub>0.7</sub> , Ile <sub>1.6</sub> , Lys <sub>1.0</sub> , Arg <sub>1.0</sub>	?
-----	-----	-----
AIIf <sub>1</sub>	CySO <sub>3</sub> H <sub>1.1</sub> , Asp <sub>2.1</sub> , Arg <sub>0.6</sub>	Arg-CySO <sub>3</sub> H-Asp-Asp
AIIf <sub>2</sub>	CySO <sub>3</sub> H <sub>2.7</sub> , Asp <sub>4.0</sub> , Thr <sub>3.0</sub> , Ser <sub>1.6</sub> , Glu <sub>1.1</sub> , Gly <sub>3.0</sub> , Val <sub>0.8</sub> , Ile <sub>1.7</sub> , Lys <sub>1.0</sub> , Arg <sub>2.0</sub>	?
AIIf <sub>3</sub>	CySO <sub>3</sub> H <sub>2.4</sub> , Asp <sub>2.0</sub> , Thr <sub>3.0</sub> , Ser <sub>1.5</sub> , Glu <sub>1.7</sub> , Gly <sub>2.0</sub> , Ile <sub>0.7</sub> , Leu <sub>1.0</sub> , Arg <sub>0.9</sub>	?
-----	-----	-----
AIVa	CySO <sub>3</sub> H <sub>1.0</sub> , Thr <sub>4.0</sub> , Ser <sub>1.0</sub> , Pro <sub>1.0</sub> , Gly <sub>1.0</sub>	Thr-Pro-Thr-Thr-Thr-
AIVb	CySO <sub>3</sub> H <sub>1.0</sub> , Gly <sub>1.5</sub> , Arg <sub>0.9</sub>	Gly-CySO <sub>3</sub> H-Ser Arg-Gly-CySO <sub>3</sub> H-(Gly)
-----	-----	-----
AVa	CySO <sub>3</sub> H <sub>1.0</sub> , Glu <sub>0.9</sub> , Leu <sub>0.8</sub> , His <sub>1.0</sub>	Leu-Glu-CySO <sub>3</sub> H-His
AVb	CySO <sub>3</sub> H <sub>1.0</sub> , Tyr <sub>1.0</sub> , Lys <sub>1.0</sub>	CySO <sub>3</sub> H-Tyr-Lys
AVc	CySO <sub>3</sub> H <sub>1.0</sub> , Tyr <sub>1.0</sub> , Lys <sub>2.0</sub>	CySO <sub>3</sub> H-Tyr-Lys-Lys
AVd	CySO <sub>3</sub> H <sub>1.0</sub> , Tyr <sub>1.0</sub> , Lys <sub>2.0</sub> , Arg <sub>1.0</sub> , ox. Trp	CySO <sub>3</sub> H-Tyr-Lys-Lys- ox. Trp

## APPENDIX A-2

Table II

Amino acid composition and probably sequence of cysteic acid peptides obtained from acid hydrolysates of the double cystine peptide AIIa

Peptides	Amino acid composition	Probable sequence of the peptides
PII <sub>6</sub>	CySO <sub>3</sub> H 1.0, Asp 1.0	Asp-CySO <sub>3</sub> H
PII <sub>5</sub>	CySO <sub>3</sub> H 1.0, Asp 1.0, Ile 0.7	Ile-Asp-CySO <sub>3</sub> H
PII <sub>4</sub>	CySO <sub>3</sub> H 1.0, Asp 1.2, Glu 1.0, Ile 1.0	Glu-Ile-Asp-CySO <sub>3</sub> H
PII <sub>3</sub>	CySO <sub>3</sub> H 1.0, Gly 0.9	Gly-CySO <sub>3</sub> H
PIII <sub>5</sub>	CySO <sub>3</sub> H 2.4, Asp 1.0	CySO <sub>3</sub> H + CySO <sub>3</sub> H-Asp
PIII <sub>4</sub>	CySO <sub>3</sub> H 2.0, Asp 1.0, Glu 0.7, Ile 2.0	Ile-Glu-Ile-Asn-CySO <sub>3</sub> H-CySO <sub>3</sub> H
PIII <sub>3</sub>	CySO <sub>3</sub> H 1.0, Asp 1.0	CySO <sub>3</sub> H-Asn
PIII <sub>2</sub>	CySO <sub>3</sub> H 1.0, Asp 1.0, Glu 1.0, Ile 0.9	Glu-Ile-Asn-CySO <sub>3</sub> H
PIII <sub>1</sub>	CySO <sub>3</sub> H 1.0, Asp 1.0, Glu 1.0, Gly 0.9, Ile 2.0	Gly-Ile-Glu-Ile-Asn-CySO <sub>3</sub> H
PIV <sub>5</sub>	CySO <sub>3</sub> H 2.0, Asp 0.6	Asp-CySO <sub>3</sub> H-CySO <sub>3</sub> H
PIV <sub>4</sub>	CySO <sub>3</sub> H 2.0, Asp 1.0, Glu 0.7, Ile 1.2	Ile-Glu-Ile-Asp-CySO <sub>3</sub> H-CySO <sub>3</sub> H
PIV <sub>3</sub>	CySO <sub>3</sub> H 2.0, Asp 1.0, Glu 1.0, Gly 0.4, Ile 2.0	Gly-Ile-Glu-Ile-Asp-CySO <sub>3</sub> H-CySO <sub>3</sub> H
PIV <sub>2</sub>	CySO <sub>3</sub> H 1.0, Asp 1.0	CySO <sub>3</sub> H
PIV <sub>1</sub>	CySO <sub>3</sub> H 2.1, Asp 2.0, Pro 1.0, Gly 1.0	CySO <sub>3</sub> H-Asn
PV <sub>3</sub>	CySO <sub>3</sub> H	Gly-CySO <sub>3</sub> H-Pro + CySO <sub>3</sub> H-Asn-A
PV <sub>2</sub>	CySO <sub>3</sub> H 1.0, Gly 1.0	CySO <sub>3</sub> H
PV <sub>1</sub>	CySO <sub>3</sub> H 1.0, Pro 1.1	Gly-CySO <sub>3</sub> H
PVI <sub>1</sub>	CySO <sub>3</sub> H	CySO <sub>3</sub> H

APPENDIX B-1

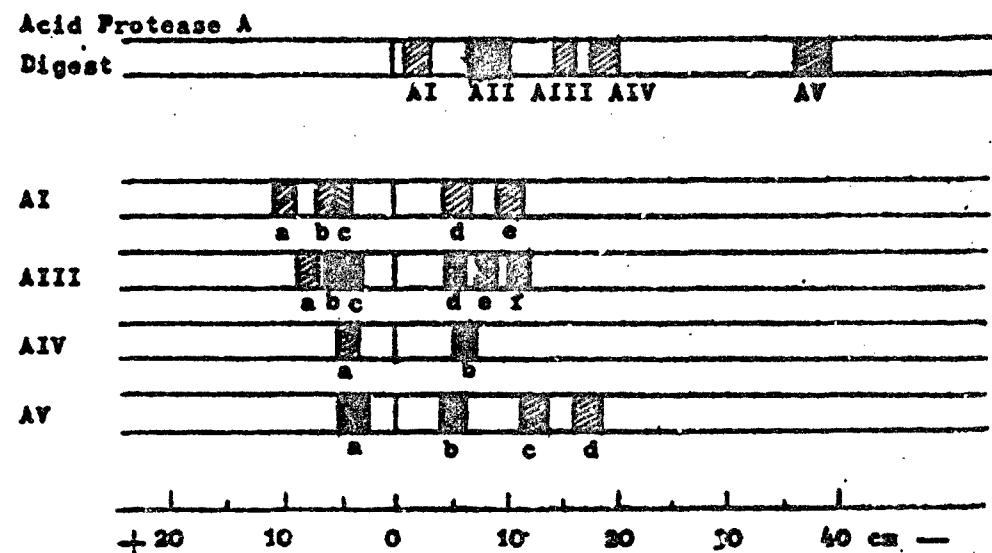


Fig. 1. Electrophoretic separation of cystine peptides from acid protease A digest of cobrotoxin (pH 5.4, 48 V/cm, 90 min) and of cysteic acid peptides from the cystine peptides (pH 5.4, 48 V/cm, 60 min).

APPENDIX B-3

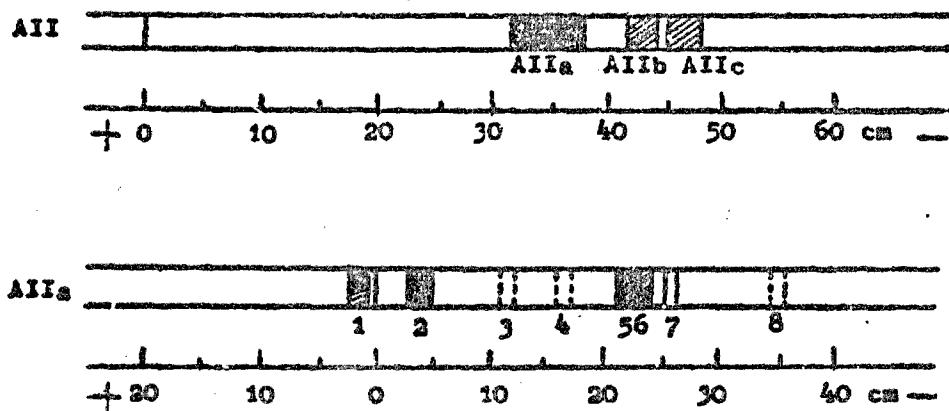
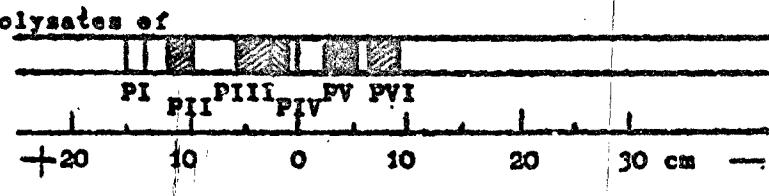


Fig. 2. Electrophoresis of the neutral band AII at pH 1.9 and the separation of cysteic acid peptides from AIIa (pH 1.9, 56 V/cm, 90 min).

APPENDIX B-3.

(A)

Acid Hydrolysates of  
AIIa



(B)

Reference

PII

PIII

PIV

PV

PVI

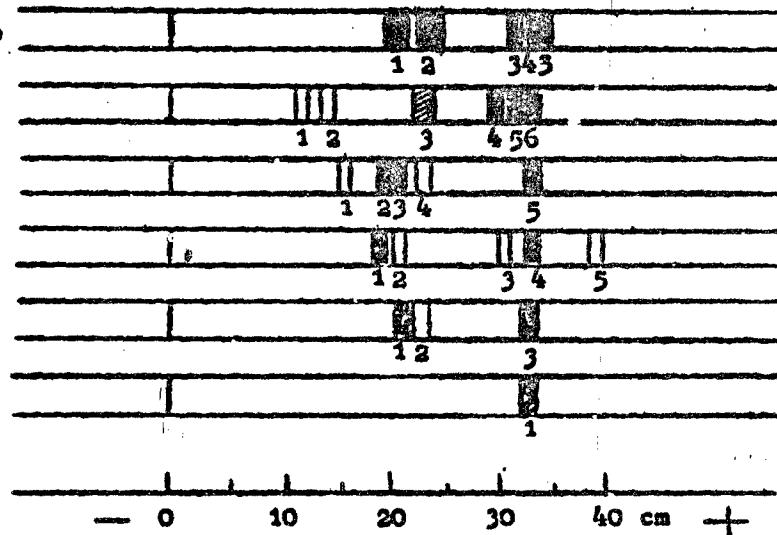


Fig. 3. Electrophoretic separation of (A) cystine peptides from acid hydrolysates of AIIa (pH 5.4, 46 V/cm, 90 min) and of (B) the oxidation products of the cystine peptides (pH 5.4, 46 V/cm, 60 min).

References: 1, Reduced GSH; 2, Glu; 3, Asp; 4, CySO<sub>3</sub>H; 5, Oxidized GSH.

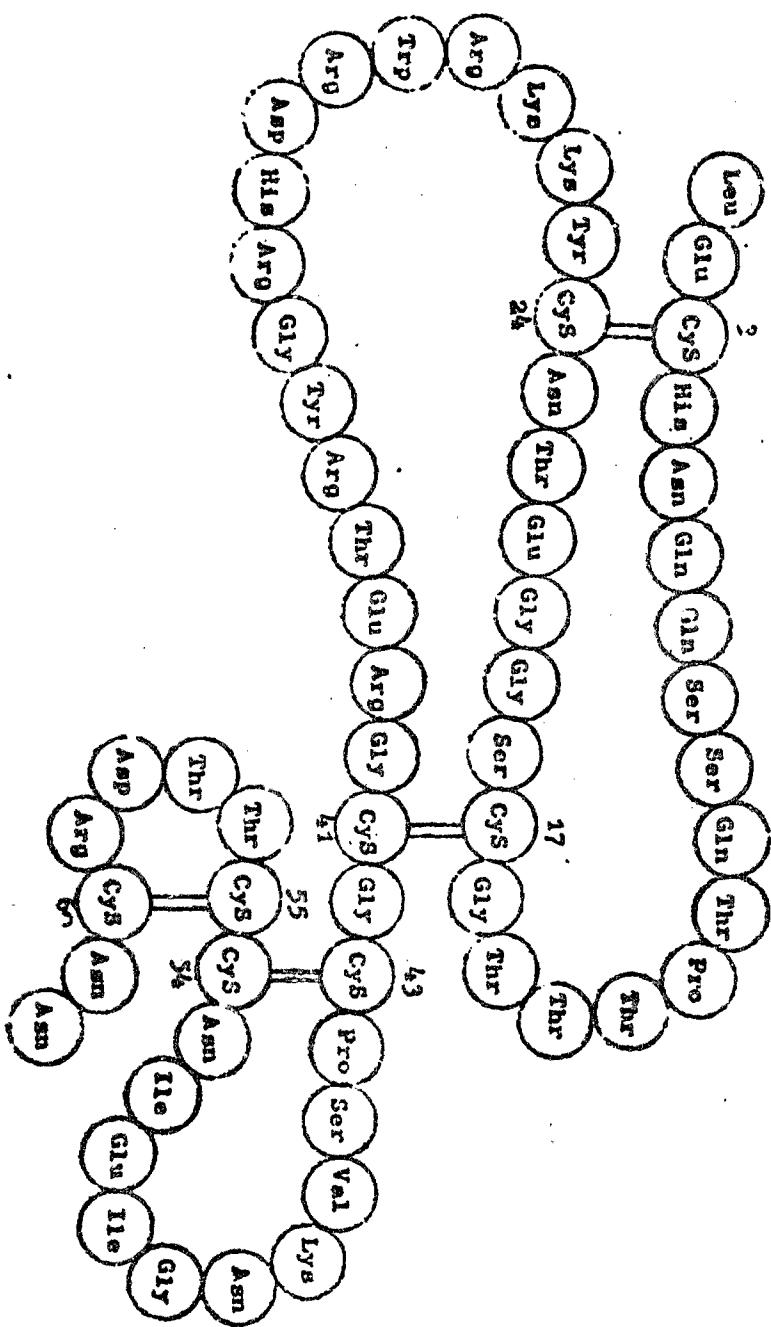


FIG. 4. Structure of cobrotoxin.

Two-dimensional schematic diagram showing the arrangement of the disulfide bonds and the sequence of the amino acid residues.

List of Publications

1. The disulfide bonds of cobrotoxin and their relationship to lethality. Biochim. Biophys. Acta, 133 (1967) 346.
2. Optical rotatory dispersion of cobrotoxin. J. Biochem., 61 (1967) 272.
3. Biochemical studies on the toxic nature of snake venom. Intern. Congr. Biochem., 7th, Tokyo Col. VIII, 1 (1967).
4. Studies on fluorescent cobrotoxin. Biochim. Biophys. Acta, 147 (1967) 600.
5. Study on  $I^{131}$  labeled cobrotoxin. Toxicon, 5 (1968) 295.
6. Optical rotatory dispersion and circular dichroism of cobrotoxin. Biochim. Biophys. Acta, 168 (1968) 373.
7. Amino acid composition and end group analysis of cobrotoxin. Toxicon, 7 (1969) 43.
8. Immunochemical studies on cobrotoxin. J. Immun., 102 (1969) 1437.
9. The amino acid sequence of cobrotoxin. Biochim. Biophys. Acta, 188 (1969) 65.
10. Biochemical and immunochemical studies on cobrotoxin. Toxicon, 8 (1970)

List of Graduate Students

Chun-chang Chang, D. Sc.

Yea-shiang Su, B.S.

Lung-hsiung Hsu, B.S.

Che-an Yang, B.S.

Hung-chih Chiu, B.S.

Unclassified  
Security Classification

**DOCUMENT CONTROL DATA - R & D**

(Security classification of title, body of abstract and indexing annotation must be entered when the overall report is classified)

1. ORIGINATING ACTIVITY (Corporate author) Department of Biochemistry Kaohsing Medical College Kaohsing, Taiwan		2a. REPORT SECURITY CLASSIFICATION Unclassified
		2a. GROUP
3. REPORT TITLE  STRUCTURE-ACTIVITY RELATIONSHIPS AND IMMUNOCHEMICAL STUDIES ON COBROTOXIN (U)		
4. DESCRIPTIVE NOTES (Type of report and inclusive dates) Final Report No. 2, 15 July 1969 - 14 July 1970		
5. AUTHOR(S) (First name, middle initial, last name)  Chen-chung Yang		
6. REPORT DATE 10 July 1970	7a. TOTAL NO. OF PAGES 17	7b. NO. OF REFS 21
8a. CONTRACT OR GRANT NO. DA-CRD-AFE-S92-544-69-GL53	8b. ORIGINATOR'S REPORT NUMBER(S) FE-428-2	
8c. PROJECT NO. 2N061102B71D	8d. OTHER REPORT NO(S) (Any other numbers that may be assigned this report)	
8e. Task 00 128FE 4		
9. DISTRIBUTION STATEMENT  This document has been approved for public release and sale; its distribution is unlimited.		
10. SUPPLEMENTARY NOTES	12. SPONSORING MILITARY ACTIVITY  U.S. Army R&D Group (Far East) APO San Francisco 96343	

The amino acid sequence of cobrotoxin has recently been established in our laboratory. However, in consideration that the disulfide bonds are the major bonds which maintain the protein in its native configuration and that the integrity of the disulfide bonds is important for the venom toxicity, it is therefore essential that each pair of the half-cystinyl residues is determined, to assist in the elucidation of the two-dimensional structure of cobrotoxin.

The fragmentation of protein in the study of disulfide bridges is usually carried out with pepsin under acidic pH. However, cobrotoxin is not attacked by pepsin and is also not cleaved to single cystine peptides by either trypsin or chymotrypsin or the combination of both. Consequently, the fragmentation of the toxin was carried out with acid protease A.

Cobrotoxin was digested with acid protease A in 5% acetic acid and the resulting S-containing peptides were separated by high-voltage paper electrophoresis. The identification of the disulfide bridges was made by determining the amino acid composition of the corresponding cysteic acid peptides obtained after the oxidation of the single cystine peptides. However, peptide AII gave the result indicating that it represents the sequence from positions (42)-43 to 61-(62) of cobrotoxin. Hence it is a double cystine peptide which contains the -CyS-CyS- linkage in sequence was not (Con'd)

DD FORM 1473  
1 SEP 60  
REPLACES DD FORM 1473, 1 JAN 64, WHICH IS  
OBsolete FOR ARMY USE.

~~Unclassified~~  
~~Security Classification~~

cleaved by the acid protease A. Therefore, the peptide was further subjected to partial acid hydrolysis at 37° for 10 days with 10 N  $H_2SO_4$  in 50 % acetic acid containing thioglycollic acid. Five cystine peptides were obtained from which the two remaining disulfide bridges were established.

The result of this study was summarized in Fig. 4, which gave the complete structure of cobrotoxin showing the position of the disulfide bonds and the sequence of the amino acid residues. The region of the molecule from residues 25 to 40 contains most of the basic and the functional residues in close order and is completely free of prolyl and cystinyl residues. This uncross-linked loop, projecting outward from the molecule because of its hydrophilic character. It is the only region in the molecule where potentially, a considerable degree of  $\alpha$ -helical structure could be present and may play an important role in toxicity. (Author)

Unclassified

Security Classification

14. KEY WORDS	LINK A		LINK B		LINK C	
	ROLE	WT	ROLE	WT	ROLE	WT
Cobrotoxin Structure Isolation Amino acid Venom Toxicity Electrophoresis Peptide Oxidation Amino Acid Analysis Formosa						

Unclassified

Security Classification